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Targeting *Enterococcus faecalis* Biofilms with Phage Therapy

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Phage therapy has been proven to be more effective, in some cases, than conventional antibiotics, especially regarding multidrug-resistant biofilm infections. The objective here was to isolate an anti-*Enterococcus faecalis* bacteriophage and to evaluate its efficacy against planktonic and biofilm cultures. *E. faecalis* is an important pathogen found in many infections, including endocarditis and persistent infections associated with root canal treatment failure. The difficulty in *E. faecalis* treatment has been attributed to the lack of anti-infective strategies to eradicate its biofilm and to the frequent emergence of multidrug-resistant strains. To this end, an anti-*E. faecalis* and *E. faecium* phage, termed EFDG1, was isolated from sewage effluents. The phage was visualized by electron microscopy. EFDG1 coding sequences and phylogeny were determined by whole genome sequencing (GenBank accession number KP339049), revealing it belongs to the *Spounavirinae* subfamily of the *Myoviridae* phages, which includes promising candidates for therapy against Gram-positive pathogens. This analysis also showed that the EFDG1 genome does not contain apparent harmful genes. EFDG1 antibacterial efficacy was evaluated *in vitro* against planktonic and biofilm cultures, showing effective lytic activity against various *E. faecalis* and *E. faecium* isolates, regardless of their antibiotic resistance profile. In addition, EFDG1 efficiently prevented *ex vivo E. faecalis* root canal infection. These findings suggest that phage therapy using EFDG1 might be efficacious to prevent *E. faecalis* infection after root canal treatment.

Enterococcus faecalis is a commensal Gram-positive microorganism inhabiting the gastrointestinal tract. Nonetheless, it can cause life-threatening infections such as endocarditis (1), bacteremia (2), urinary tract infection, and meningitis (3), and it appears especially in hospitals where resistance to antibiotics is developed (4). In addition, *E. faecalis* is frequently recovered from secondary persistent infections associated with root canal treatment failures (5, 6) that can result in invasion to the tissues surrounding the tip of the tooth-root (periradicular tissue) with subsequent development of abscesses and diffused infections (cellulitis) (7). Moreover, despite meticulous mechanical preparation during root canal treatment, infection may persist in 20 to 33% of the root canals (8). The frustrating rates of posttreatment disease are mainly attributed to the limitations of the present technologies, which offer no tools to combat intracanal *E. faecalis* biofilm infection (5, 6).

Biofilms may pose a severe health threat, since at this phase bacteria not only become inaccessible to antibacterial agents and the body's immune system but also provide a reservoir of bacteria for chronic infections throughout the body (9). Most biofilm-associated infections, such as implant-related infections (10), oral infections (11), device-related infections, and chronic infections (such as lung infections in cystic fibrosis patients) (12) are treated today using antibiotics, for lack of a better alternative. The extensive use or misuse of antibiotics has led to an alarming emergence of virulent, antibiotic-resistant pathogenic bacteria (13). Moreover, it is well established that attacking mature biofilms with conventional antibiotics works poorly, requiring much higher drug doses than usual (9). The penetration failure may be associated with various factors, including the extracellular sheath, multidrug resistance development of bacteria within the biofilm (14, 15), cell cluster mode of action (16, 17), and "bet-hedging" strategies in bacterial cultures such as programmed-cell-death that provide nutrients for the community and DNA for the biofilm matrix (18). This challenge calls for different measures of antimicrobial protection: one that delivers an antimicrobial agent to incapacitate biofilm-forming bacteria and one that prevents the proliferation of bacteria in biofilms. Consequently, the development of new antimicrobial agents has become paramount (14).

One alternative recently regaining interest is bacteriophage (phage) therapy (19, 20), which was first introduced by Felix d'Herelle at the beginning of the 20th century. Historically, it was successfully used in western countries (21, 22) and abandoned with the emergence of antibiotics. Nonetheless, it is in use until today in eastern European countries (21, 23). The key benefits of phage therapy (24) are as follows: (i) their relative specificity, which is less likely to impact the commensal flora; (ii) their ability to multiply at the infection site and disappear together with the pathogen; (iii) their efficacy against biofilms; and (iv) being natural products, they are likely to be devoid of apparent toxicity. Ironically, because antibiotics were considered to be wonder

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TABLE 1 Bacterial strains and their sensitivity to EFDG1

Bacterial strain[a]	Origin[b]	EFDG1[c]	Antibiotic(s)	
			Resistance	Sensitivity
Enterococcus strains				
E. faecalis (*v583*)	ATCC 700802	S	Vancomycin, gentamicin	Daptomycin, streptomycin
E. faecalis (*aef01*)	Clinically isolated from urine	S		Ampicillin, ciprofloxacin, nitrofuantoin, vancomycin
E. faecalis (*aef03*)	Clinically isolated from urine	S		Ampicillin, ciprofloxacin, nitrofuantoin, vancomycin
E. faecalis (*aef04*)	Clinically isolated from venal blood flow	S	Erythromycin	Ampicillin, ciprofloxacin, vancomycin
E. faecalis (*aef05*)	Clinically isolated from venal blood flow	S	Erythromycin, gentamicin	Ampicillin, chloramphenicol, vancomycin
E. faecalis (*cef02*)	Clinically isolated	S		
E. faecium (*aefc06*)	Clinically isolated from venal blood flow	S	Ampicillin, erythromycin, vancomycin, gentamicin, streptomycin	Chloramphenicol
E. faecium (*aefc07*)	Clinically isolated from venal blood flow	S	Ampicillin, erythromycin, ciprofloxacin, gentamicin	Vancomycin
E. faecium (*aefc08*)	Clinically isolated from venal blood flow	S	Gentamicin, streptomycin	
E. faecium (*aefc09*)	Clinically isolated from feces	S	Vancomycin	
E. faecium (*aefc10*)	Clinically isolated from feces	S	Vancomycin	
Staphylococcus strains				
S. aureus (*w6460*)	Clinically isolated	R		
S. aureus (*w0406*)	Clinically isolated	R		
S. aureus (*lsa011*)		R		
Other strains				
Pseudomonas aeruginosa PA14		R		
Pseudomonas aeruginosa PA14 *pqsA*		R		
Streptococcus mutans (*lsm012*)		R		
Streptococcus sobrinus (*lsb013*)		R		
Fusobacterium nucleatum (*fs014*)		R		
Porphyromonas gingivalis (*pg015*)		R		
Burkholderia cepacia complex 25	Clinically isolated	R		
Burkholderia cepacia complex 80	Clinically isolated	R		
Klebsiella pneumonia (*bkp016*)		R		

[a] Strains were grown in a 96-well plate reader for 72 h. EFDG1 (MOI of 0.1) was added at time zero (logarithmic) or at 24 h (stationary), and the optical density was recorded every 20 min. The anaerobes *F. nucleatum* and *P. gingivalis* were grown under anaerobic conditions, and the optical density was measured at the endpoint. Locus tags are indicated in parentheses.

[b] The clinical isolations had been performed in the Hadassah Medical Center, Jerusalem, Israel.

[c] The bacterial sensitivity of the clinical isolates to antibiotics was determined by the infectious disease unit of Hadassah Hospital, Jerusalem, Israel. S, sensitive; R, resistant.

drugs, curing deadly diseases in the past few decades, they have been used extensively, resulting in the rise of untreatable multidrug-resistant pathogens. Along with the rise of antibiotics, phage therapy quickly lost popularity due to fear of possible unknown harmful genes and the phages' unknown nature. Recently, with the emergence of multidrug resistant strains and the high-throughput sequencing abilities, the risk of using phages with unwanted genes has been greatly reduced. Phage therapy is being considered, again, for use both in the food industry and in medicine (25). In addition, several reports showed that phage therapy improved (26–28), and in some cases was even more successful than (29, 30), antibiotic treatment.

Despite the significance of *E. faecalis* in root canal infections, very few attempts were made to treat *E. faecalis* infection using phage therapy (31, 32). In the present study, an *E. faecalis*-infecting phage was isolated from sewage and characterized genetically by sequencing and functionally by evaluating its efficacy against *E. faecalis* planktonic and biofilm cultures *in vitro*. The phage's therapeutic potential was demonstrated *ex vivo* in a human root canal model.

MATERIALS AND METHODS

Bacterial strains and materials. *E. faecalis* V583 (ATCC 700802) was grown in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C under aerobic conditions with shaking at 200 rpm. Additional bacterial strains used here for screening purposes are listed in Table 1. Unless otherwise mentioned, all materials were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and propagation of phages. Isolation of phages was performed using the standard double-layered agar method (33). Briefly, sewage effluent from the West Jerusalem sewage treatment facility was centrifuged (centrifuge 5430R, rotor FA-45-24-11HS; Eppendorf) at 10,000 × *g* for 10 min, and the supernatant was filtered first through 0.45-μm-pore-size filters (Merck Millipore, Ltd., Ireland) and then through 0.22-μm-pore-size filters (Merck Millipore). Exponentially grown bacterial cultures (10^8 CFU/ml) were inoculated with filtered sewage effluent for 24 h at 37°C. The cultures were refiltered and added

to 5 ml of BHI broth containing 0.5 ml of overnight (10^9 CFU/ml) cultures of *E. faecalis*, which were incubated until complete lysis was obtained. The lysate was diluted in BHI broth, plated using soft agar (0.6%) that was overlaid with the test strain, and then incubated overnight at 37°C as described above. Plaque morphologies were observed, and clear ones were transferred into a tube of broth using a sterile Pasteur pipette. The phage stocks were replated with bacterial cultures in order to collect high titer lysates, which were then stored in BHI with chloroform (40 ml/liter) at 4°C.

The concentration of PFU was determined according to the standard method. Lysates were serially diluted 10-fold into 5 ml of prewarmed BHI soft agar (0.6%). A 0.1-ml portion of overnight culture of *E. faecalis* was added to the tube, which was placed on a BHI agar plate. The number of plaques was counted, and the initial concentration of PFU was calculated (33).

Assessment of phage lytic activity in planktonic cultures. Lytic activity was assessed by inoculating logarithmic (10^7 CFU/ml) or stationary (10^9 CFU/ml) *E. faecalis* cultures with purified phages at various multiplicity of infection (MOI) of 0, 0.01, 1, and 100 in triplicates. The growth kinetics of the cultures was recorded at 37°C with 5-s shaking every 20 min in a 96-well plate reader (Synergy; BioTek, Winooski, VT) at 600 nm. The live bacterial count was determined at the end time point by counting the CFU/ml.

Assessment of phage lytic activity in biofilm. *E. faecalis* V583 static biofilms were grown for 2 weeks in a 96-well plate at 37°C to a width of approximately 100 μm, phages were added (10^7 PFU/well), and incubation was continued for an additional week. The wells were then washed with phosphate-buffered saline, and the biomass was quantified using crystal violet staining as previously described (34). Briefly, fixation was achieved by adding methanol (200 μl) to the wells, followed by incubation for 20 min, followed in turn by methanol aspiration and air drying. The biofilms were stained by 200 μl of crystal violet (1%) for 20 min at room temperature and then washed with water. Ethanol (200 μl) was added, and biomass was quantified by determining the optical density at 538 nm (OD_{538}). In addition, the wells were stained using Live/Dead cell viability kits (Life Technologies, Waltham, MA) according to the manufacturer's instructions. The fluorescence emissions of the samples were detected by using a Zeiss LSM 410 confocal laser microscope (Carl Zeiss). Red fluorescence was measured at 630 nm, and green fluorescence was measured at 520 nm. Horizontal plane optical sections were made at 5-μm intervals from the surface outward, and the images were displayed individually. The microscopy slices were combined to a 3D image using Bio-formats and UCSD plugins (ImageJ 1.49G). The stained biofilms were examined using a confocal microscope and analyzed using ImageJ 1.49G software (http://imagej.nih.gov/ij/).

TEM visualization. For the visualization of isolated phages using transmission electron microscopy (TEM), the classic method of Gill was followed, as described in OpenWetWare (http://openwetware.org/wiki/Gill:Preparing_phage_specimens_for_TEM). According to this method, 1 ml of lysate with 10^9 PFU/ml was centrifuged at 19,283 × *g* (centrifuge 5430R, rotor FA-45-24-11HS; Eppendorf) for 2 h at room temperature. The supernatant was discarded, and the pellet was resuspended in 200 μl of 5 mM $MgSO_4$. The pellet was allowed to soak overnight in a 4°C fridge and then resuspended by gently pipetting up and down (no vortexing). A paper towel was placed on the bench, and a strip of Parafilm was placed on it; to this, 30 μl of 5 mM $MgSO_4$ and 10 μl of the phage sample was added, and the sample was mixed gently. For each of the grids to be prepared, 30 μl of 2% uranyl acetate was pipetted onto the Parafilm. The grids were then placed carefully on the drop of phage sample using forceps, with the carbon side facing down. After about a minute, the grid was placed on the drop of the stain (2% uranyl acetate), followed by incubation for about a minute. The grids were then dried and stored in the desiccator until further use. A transmission electron microscope (Joel, TEM 1400 plus) with a charge-coupled device camera (Gatan Orius 600) was used to capture images. In all of the experiments described here, significant differences were determined from a Student *t* test performed using GraphPad Prism v5.03 for Windows (GraphPad Software, San Diego, CA).

Host range specificity tests. The activity of EFDG1 was screened against clinical isolates from the infectious diseases unit of Hadassah Hospital and strains from our lab collections (Table 1). Aerobic bacterial growth kinetics were monitored using a 96-well plate reader. Anaerobic strains were grown in anaerobic jars, and their optical density was recorded every 24 h.

DNA isolation and sequencing. Phage DNA isolation was performed as previously described (33). Briefly, phages were added to an overnight *E. faecalis* culture (MOI = 0.01) and incubated for 48 h at 37°C until total bacterial clearance was observed and a high titer lysate (10^9 PFU/ml) was obtained. The culture was treated with DNase (100 mg/liter) and RNase (50 mg/liter) at 37°C for 30 min to destroy bacterial nucleic acids. Proteinase K (100 mg/liter) and sodium dodecyl sulfate (20%) were added for 1 h at 52°C to digest both phage capsid and DNase.

Sequencing was performed in the interdepartmental unit at the Hebrew University, Hadassah Campus, as described previously (35). Libraries were prepared by using a Nextera XT DNA kit (Illumina, San Diego, CA), and DNA was amplified by a limited-cycle PCR and purified using AMPure XP beads. The DNA libraries were normalized, pooled, and tagged in a common flow cell at 2×250 base-paired-end reads using the Illumina MiSeq platform. The quality of the reads was determined using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc), and reads were trimmed and cleaned by FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html).

De novo assembly, open reading frame (ORF) prediction, alignment of whole-phage genomes, and phylogenetic tree generation were performed using Geneious 7.1.5 (Biomatters). Blast, blastX, gene annotation, and gene ontology (GO) analysis were completed using Geneious 7.1.5 and blast2go (36). tRNAs were predicted by using *tRNA*scan-SE v1.21 (37).

The circular nature of EFDG1 genome was validated by PCR amplification using oligonucleotides which correspond to the flanking region of the putative "seam" (GATGGAGACACGGAAGCTGT and CGGCTTTC CCCGTATACCTC). As a control, we used oligonucleotides that amplify a fragment with high coverage, distant from the "seam" (GCCAAGCTT CTCACACTTCC and CCACCTTTTTGTCAGGTCGT).

***Ex vivo* human root canal model.** Extracted one-rooted teeth were subjected to endodontic treatment, including standard cleaning, shaping, filling, and coronal part removal by a diamond bur. Standard endodontic access to the canal was performed using Gates-Glidden drills, followed by autoclave sterilization. Canals were contaminated with *E. faecalis* suspension (250 μl from a culture with an OD_{600} of 0.1), and the root canals were prepared using K-files (Micro Mega, Besancon, France) and irrigated with 2.5% NaOCl and EDTA cream (Micro Mega) according to a standard procedure. After the third K-file shaping, the canals were recontaminated with an *E. faecalis* suspension. Final cleaning and shaping was performed by two sequential K-files, including 2.5% NaOCl irrigation and EDTA. The canals were obturated in a standard procedure using gutta-percha and an endodontic sealer (AH26; Denspaly, Constance, Germany). The phage-treated group teeth were irrigated additionally with 250 μl of phages (10^8 PFU/ml).

Bacterial leakage was assessed using a two-chamber bacterial leakage model (38). The coronal part (1 mm) of the roots was subjected to further bacterial challenge, i.e., the upper chamber of the model contained an *E. faecalis* suspension (OD_{600} of 0.01), and the lower chamber contained sterile BHI broth. To prevent bacterial transfusion between the upper and lower chambers, the gap between the root and the upper chamber was sealed using a flowable resin composite (Filtek Supreme; 3M ESPE, Minneapolis, MN), and only the apical 2-mm portion of the root was placed in the lower-chamber sterile BHI broth. The turbidity was assessed every 24 h, and samples were plated to determine the number of live bacteria (CFU/ml). The roots were then horizontally split in the center, and the internal part of each root was dyed by using a Live/Dead cell viability kit

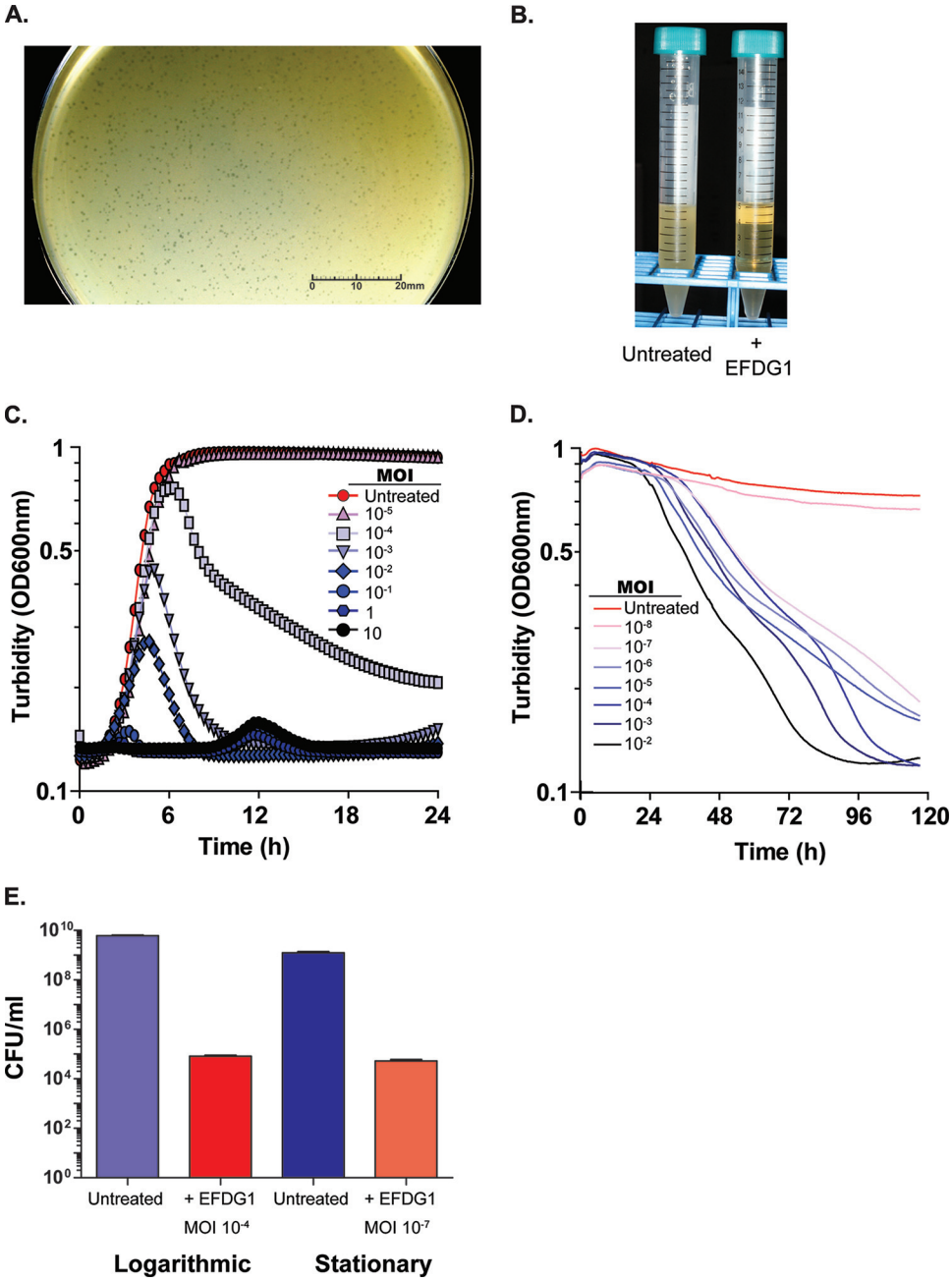

FIG 1 EFDG1 is an efficient lytic phage that infects *E. faecalis*. (A) Clear plaques of EFDG1 grown on an *E. faecalis* lawn. (B) *E. faecalis* overnight culture was diluted to 1:1,000 in the absence or presence of EFDG1 phages (MOI = 10^{-4}), followed by incubation for 24 h. Total clearance is observed in the treated culture. (C) EFDG1 kills logarithmic *E. faecalis* in a dose-dependent manner, with an MOI as low as 10^{-4}. Bacterial cultures were grown as in panel B, and the turbidity was measured. (D) EFDG1 effectively reduces stationary cultures of *E. faecalis* at an MOI as low as 10^{-7}. The results presented in panels B and C are the averages of six independent wells. (E) Validation of the killing by CFU count of *E. faecalis* bacteria after 24 h (logarithmic, left panel) and 120 h (stationary, right panel) with or without treatment by EFDG1 at MOIs of 10^{-4} and 10^{-7}, respectively. Bars represent the average of triplicates, and error bars denote the standard deviations.

according to the manufacturer's instructions. The samples were visualized with confocal microscopy.

Genome sequence accession number. The EFDG1 genomic sequence reported here is available in the NCBI GenBank database under accession number KP339049.

RESULTS

Isolation and determination of EFDG1 efficacy against *E. faecalis* liquid cultures. *E. faecalis* phages were isolated from sewage water. The phage with the best lytic activity was termed EFDG1 (Fig. 1). This phage displayed clear plaques on double-layer agar plates (Fig. 1A) and complete lysis within 24 h in liquid culture (Fig. 1B). Quantitative analysis of EFDG1 against a logarithmic-phase culture showed that it is effective in MOIs above 10^{-4} (Fig. 1C). At MOIs of 10^{-2} to 10^{-4}, slight culture growth was observed, followed by quick lysis, and at MOIs of $>10^{-2}$ EFDG1 almost completely prevented bacterial growth.

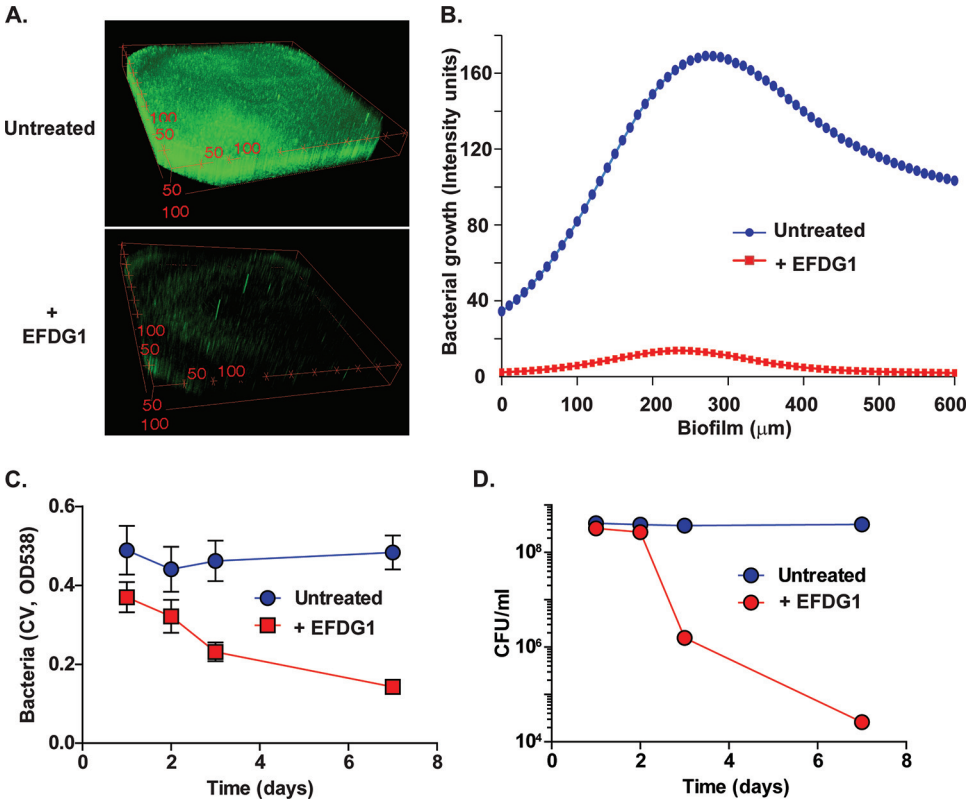

FIG 2 EFDG1 significantly reduces *E. faecalis* biofilms. EFDG1 was added to a 2-week-old biofilm of *E. faecalis*. (A) A confocal 3D image demonstrates that the phage reduces the biofilm almost completely. (B) Quantitative representation of bacteria number within the biofilm layer, as detected by confocal microscopy. These results were also validated, and the kinetics of lysis were determined by crystal violet (CV) staining (C) and a CFU count (D). Tests were performed in triplicates, and all tests yielded significant difference between treated and control groups ($P < 0.01$). Error bars denote the standard deviations.

Stationary bacterial cell elimination by EFDG1 was slower than the elimination of cells in their logarithmic phase. Nevertheless, killing was achieved even at an MOI of 10^{-7}; in other words, 100 PFU/ml were enough to eliminate 10^9 CFU of *E. faecalis* cells/ml (Fig. 1D). These results were validated by an endpoint CFU count of the lowest effective MOIs in each case, i.e., 10^{-4} after 24 h with logarithmic cells and 10^{-7} after 120 h with stationary cells, demonstrating that the number of viable *E. faecalis* cells showed a 5-log decrease after treatment with EFDG1 (Fig. 1E).

Determination of EFDG1 efficacy against biofilms of *E. faecalis*. One of the challenging pitfalls of conventional antibiotics is their limited effect against cells within bacterial biofilms, which mechanically and physiologically are less sensitive than planktonic cells (39). In contrast, EFDG1 reduced significantly and dispersed a 2-week-old 600-μm-width *E. faecalis* biofilm (Fig. 2A and B). Biofilm biomass evaluation using crystal violet showed a 5-fold reduction in the treated samples within 7 days, whereas the untreated biofilms were stable, and no reduction was observed (Fig. 2C). Viable counts showed a 5-log reduction after exposure to EFDG1, whereas no significant change was seen in the untreated biofilms (Fig. 2D). These results show that EFDG1 is a promising candidate for phage therapy against planktonic and well-established *E. faecalis* biofilms.

Assessment of EFDG1 host range of infection. The infectivity of EFDG1 was assessed on a range of aerobic and anaerobic Gram-negative and -positive bacteria. Table 1 denotes the details of the tested bacteria, including their antibiotic resistance. EFDG1 was found to be host specific, infecting only *E. faecalis* and the related *E. faecium* strains regardless of their antibiotic sensitivity. Thus far, we have not found any *E. faecalis* or *E. faecium* strains that are resistant to EFDG1.

Characterization of EFDG1 genome sequence and phylogeny. TEM microscopy showed that EFDG1 has a hexagonal head with a measured diameter of 98.71 ± 8.88 nm and tail length of 118.05 ± 6.87 nm (Fig. 3A). We performed whole-genome sequencing of EFDG1 (GenBank accession number KP339049), yielding 634,614 paired-end reads with a mean length of 244.4 ± 15.6 bp, which were trimmed and cleaned. Reads that aligned to the *E. faecalis* V583 genome (GenBank accession number AE016830) or its three plasmids (GenBank accession numbers AE016831, AE016832, and AE016833) were excluded from the analysis. The remaining reads ($n = 194{,}186$) were subjected to *de novo* assembly, which yielded 10 contigs with more than 10 reads each.

The largest and most significant contig contained 149,589 bp, assembled from 186,686 reads (96% of the reads), with a pairwise identity of 99% and a mean coverage of 295 ± 81, which was predicted to be circular (Fig. 3B). Indeed, the sequence of the PCR product fragment amplified using oligonucleotide from both "tails" of the genome confirmed that the EFDG1 genome is circular (see Fig. S1 in the supplemental material).

The EFDG1 genome is AT-rich with a GC content of 37.1%, similar to that of its host *E. faecalis* (37.5%). A BLAST search

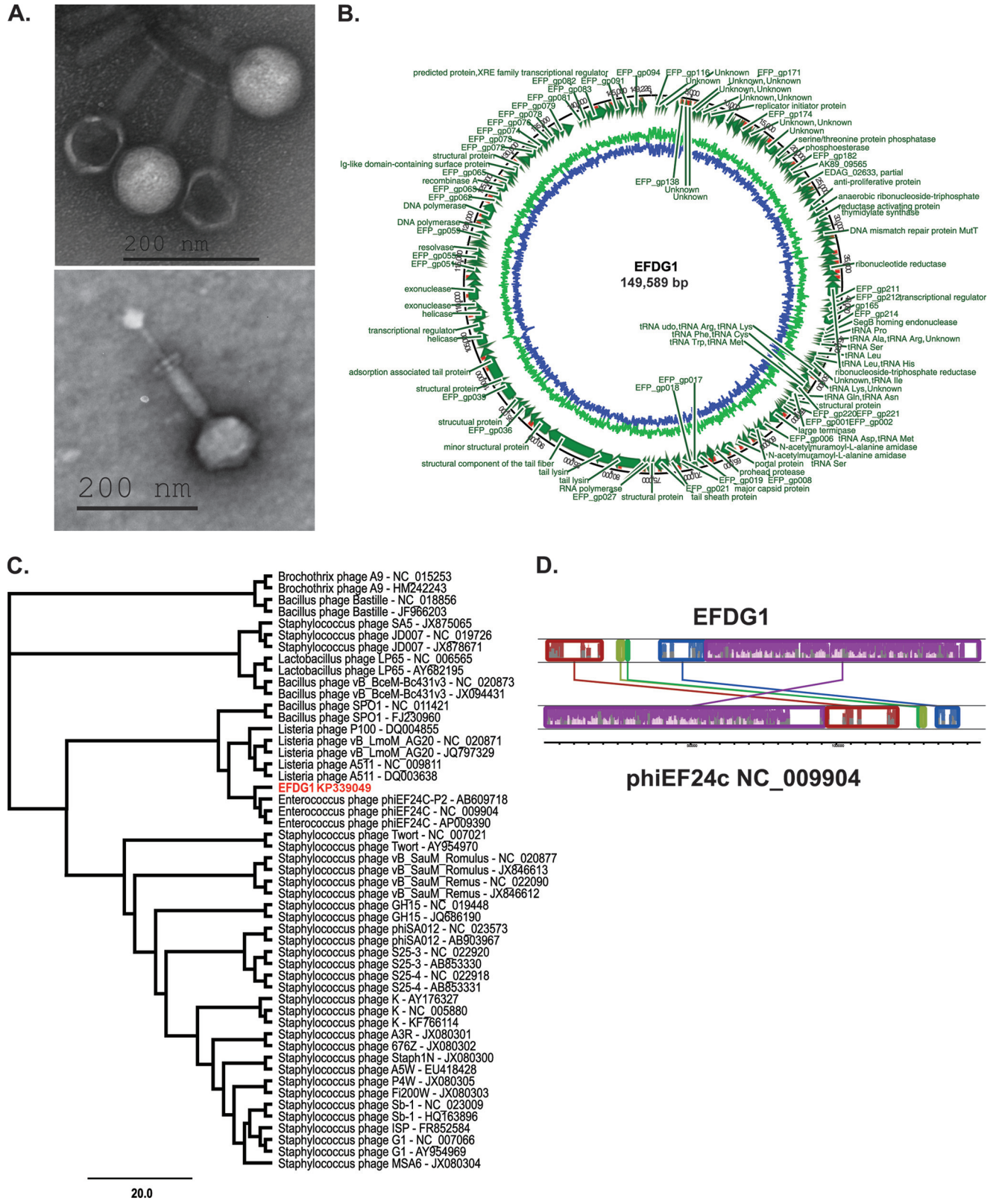

FIG 3 Characterization of EFDG1. (A) TEM of EFDG1 depicting a hexagonal head diameter of 98.71 ± 8.88 nm and a tail length118.05 ± 6.87 nm. (B to D) EFDG1 belongs to the *Spounavirinae* subfamily of the *Myoviridae* phages. (B) Schematic representation of the EFDG1 DNA sequence and putative genes (green arrows). Red squares denote repeat sequences. The graphs show the GC (blue) and AT (green) content. (C) Phylogenetic tree of EFDG1 (in red) in relation to the genomes of fully sequenced *Spounavirinae* phages. The name of the phages and their accession numbers in the NCBI database are denoted. (D) Comparison of the EFDG1 genome and its closest related phage, phiEF24c, using the Mauve plugin of Geneious 7.5.1. Similarly colored boxes indicate similar regions.

showed that EFDG1 belongs to the *Spounavirinae* subfamily (http://viralzone.expasy.org/all_by_protein/2777.html) of the *Myoviridae* family (phages with contractile tails [http://viralzone.expasy.org/all_by_protein/140.html]) of the *Caudovirales* order (tailed phages), also known as SPO1-related bacteriophages (40). Thus far, the *Spounavirinae* subfamily contains 50 members with fully sequenced genomes, all of which are Gram-positive bacterial phages, including *Staphylococcus*, *Listeria*, and *Enterococcus* phages (http://www.ebi.ac.uk/genomes/phage.html). Multiple alignment and phylogenetic tree analyses of the *Spounavirinae* phage genomes, including EFDG1 (Fig. 3C), showed that the closest phages to EFDG1 belong to the *E. faecalis* phage phiEF24c group (Fig. 3D), *Listeria* phage A511 (41), and *Staphylococcus* phage 676Z, with 55,730 (37%), 53,730 (36%), and 50,604 (34%) identical base pairs, respectively.

Prediction of ORFs larger than 100 bp identified 210 putative coding sequences and 24 tRNAs genes (GenBank accession number KP339049; see Table S1 in the supplemental material). BLASTX analysis showed that 166 (79%) of them have similarities to sequences in the nonredundant NCBI database, most of them to the phiEF24c phage (42). Putative functions could be attributed to 79 of the 166 ORFs, with the majority of them belonging to four groups. The first are phage structural genes encoding capsid and tail proteins, and proteins which are involved in adsorption and/or lysis of the host bacterial cell. The second group comprised of a large group of 16 putative proteins involved in DNA metabolism. It appears that EFDG1 contains functional DNA replication and repair machinery that includes two DNA polymerases, two exonucleases, and two helicases, as well as recombinase and resolvase (GenBank accession number KP339049; see Table S1 in the supplemental material). In addition, the EFDG1 genome contains RNA polymerase and a large set of tRNAs genes. Our analysis using Blast2Go (43) did not identify any genes known to be harmful or antibiotic resistance genes.

In addition to the annotated genes, there are 85 ORFs that are conserved and appear in other, mainly phage, genomes but do not have attributed functions. Lastly, EFDG1 has 59 ORFs that are putative coding sequences unique to this phage without any homolog in the nonredundant database. In addition to its ORFs, the EFDG1 genome contains 63 regions of repeats (Fig. 3B, red boxes), which can probably be attributed to the genome rearrangement and the differences between EFDG1 and phiEF24c.

Anti-*E. faecalis* activity in an *Ex vivo* human root canal model. To test the activity of EFDG1 in posttreated root canal infections, we used an *ex vivo* two chamber bacterial leakage model of human teeth (Fig. 4A) (38).

No turbidity was observed in the phage-treated samples, therefore, we conclude that the obturated root canals that were subjected to EFDG1 irrigation resulted in reduced bacterial leakage from the root apex compared to the control group (Fig. 4B). Indeed, quantification of viability of *E. faecalis* revealed an approximately 7-log reduction after phage irrigation (Fig. 4C).

Confocal laser scanning microscopy images of horizontal root sections showed that stained bacteria were evident only in the dentinal tubules of the group that was treated with *E. faecalis*. In contrast, no stained bacteria were seen in the phage-treated teeth or in the sterile control teeth, demonstrating the significant reduction of stained bacteria by EFDG1 (Fig. 4D).

DISCUSSION

We characterized EFDG1, a lytic phage that efficiently infects and kills planktonic and biofilm cultures of *E. faecalis* both *in vitro* and *ex vivo* in an experimental model of tooth root canal infection. The EFDG1 genome does not carry any known virulence genes such as toxins or antibiotic resistance genes that can be found in some other phages of *E. faecalis* (44). In planktonic cultures and at high MOIs, EFDG1 totally prevented culture growth and resulted in a 5-log growth reduction in stationary cultures (Fig. 1E). Moreover, EFDG1 significantly reduced a 2-week-old biofilm, demonstrating the superiority of phage therapy over conventional antibiotic treatments in biofilms, which are considered a major pitfall of antibiotics (39).

According to its genome sequence, EFDG1 belongs to the *Spounavirinae* subfamily of tailed phages, also referred to as SPO1-like and Twort-like phages, (http://viralzone.expasy.org/all_by_protein/2777.html, http://www.ebi.ac.uk/ena/data/view/Taxon:857473). These phages are lytic, infecting Gram-positive bacteria, and harbor a 130- to 160-kb linear or circular genome encoding 190 to 230 proteins. The length of their contractile tail is between 150 and 240 nm, and it contains at its tip globular structures of a double base plate, six long terminal fibers, and six short spikes. Their capsid heads are composed of protomers with a T=16 symmetry and a diameter of 85 to 95 nm.

Due to their efficient obligatorily lytic nature, the *Spounavirinae* phages seem to be promising candidates for phage therapy against Gram-positive pathogens (45). Indeed, some of them have already been suggested as therapeutic agents to control bacterial infections, e.g., P100 phage of *Listeria* spp. (46), phage K (47), Romulus and Remus (48) and other Twort-like phages (45) of *S. aureus*, the ACT group of *Bacillus* (49), and phiEF24c of *E. faecalis* (50). The latter, phiEF24c, which was isolated in Japan, is thus far the most studied *E. faecalis Spounavirinae* phage (42, 50–52). This phage, which is close in genome size, GC content, and sequence to EFDG1, was found to protect mice from *E. faecalis* infection in the abdomen and was not harmful to the mice *per se*. These previous findings in other related phages support the notion that EFDG1 is a promising candidate for phage therapy against *E. faecalis* in root canal infections.

According to our results (Table 1) the host range of EFDG1 is limited to *E. faecalis* and to *E. faecium*, a closely related bacterium. Such high host specificity is common among phages and is another advantage as phages do not tend to harm the natural beneficial microbiome as antibiotics often do.

EFDG1 was found to be an efficient killer of *E. faecalis* in an *ex vivo* model of root canal infection (53), where it reduced significantly the levels of *E. faecalis*, a bacterium known to be less susceptible to antimicrobials in the presence of dentin (47). The usage of this *ex vivo* microleakage model of human extracted teeth has many advantages, including the ability to monitor and quantify treatment outcomes in a comparable way to *in vitro* models and to simultaneously perform the examination in an anatomically and histologically similar milieu to *in vivo* conditions (53).

In summary, our results support the notion that bacteriophages can be easily isolated and characterized and that phage therapy, when used with caution (54), is a promising complementary strategy to conventional antibiotic treatment, especially when treatment fails, e.g., in the case of biofilm and multidrug resistance strains. In the particular case of *E. faecalis*, we demonstrate that

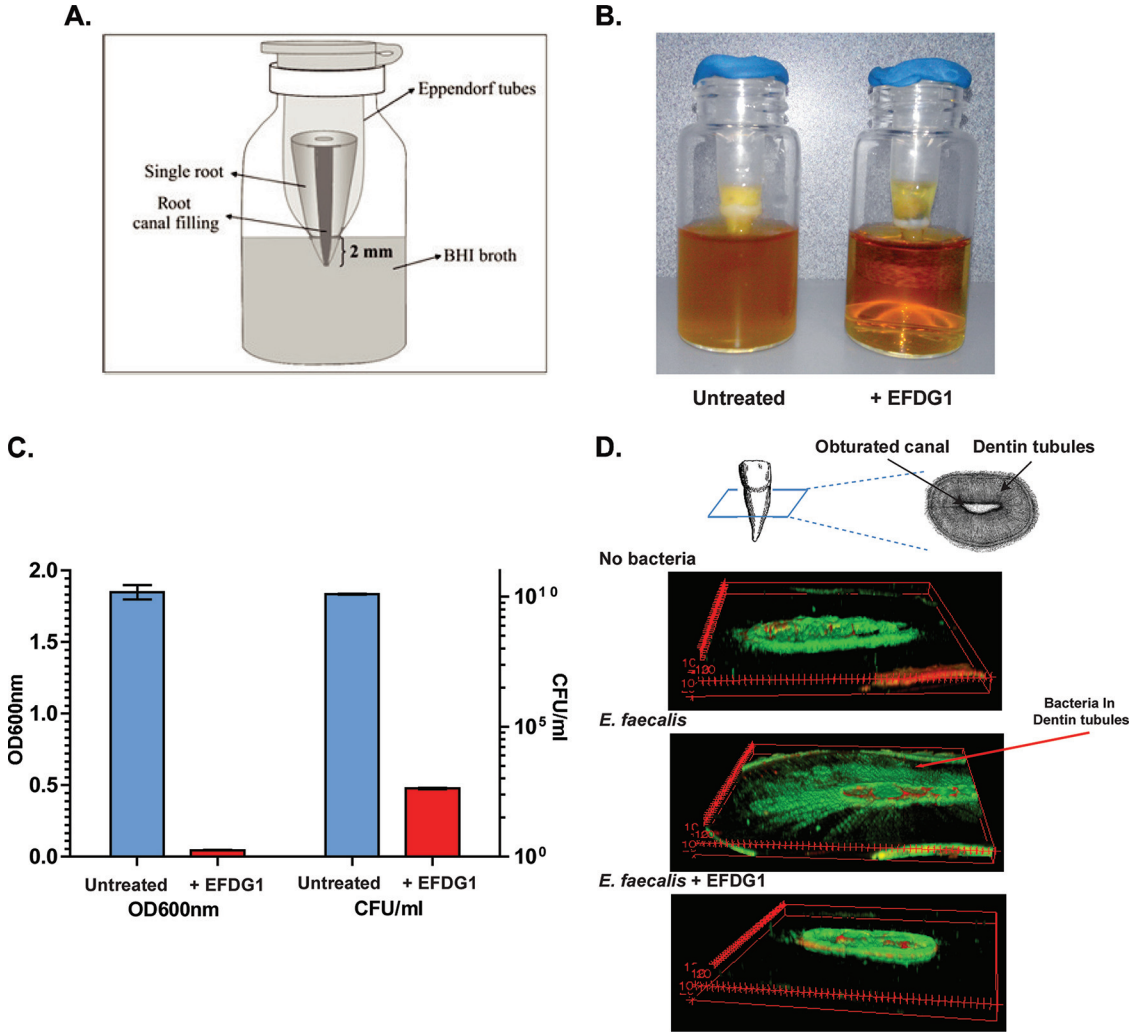

FIG 4 EFDG1 protects root canals from infection. (A) Schematic depicting the *ex vivo* root canal treatment model. One-rooted human teeth were subjected to endodontic treatment, including standard cleaning, shaping, and filling. Bacterial contamination was performed before, during, and after the root canal treatment. The test group included phage irrigation in addition to the standard procedure. (B) Photographic depiction of the root irrigated with EFDG1 phage showing clear broth in the lower chamber (48 h), indicating no bacterial outgrowth (right), and control root subjected to the standard protocol, demonstrating broth turbidity (left). (C) OD_{600} (left axis) and number of live bacteria by CFU count (CFU/ml, right axis) in the lower chamber. Viable *E. faecalis* counts depicted were reduced by at least 8 logs following phage irrigation. All experiments were repeated at least three times independently, and error bars denote standard deviations. (D) Confocal fluorescence microscopy images of a horizontal root section of the phage treated tooth depicting low numbers of stained bacteria compared to the control, where stained live and dead bacteria are depicted in the dentinal tubules surrounding the root canal (red arrow). Note that the root canal is stained nonspecifically, even in the absence of bacteria.

EFDG1 is a candidate for phage therapy against problematic *E. faecalis* infections after root canal treatment and perhaps also in endocarditis and other infections of *E. faecalis*.

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